Research Proposal

Contact Chemosensation and Neural Control of Internal States in *Drosophila melanogaster*

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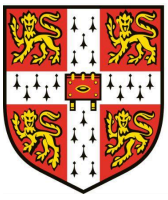

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Summary

During social behaviour organisms adapt to cues from their interaction partners. Such cues contribute to internal states which ensure that appropriate behaviours are produced. Despite their importance, the precise neural mechanisms underlying the induction of such states remain poorly understood.

Drosophila courtship is an excellent framework for studying internal states, with males performing stereotypical behaviour upon detection of a female. P1 neurons are central to this courtship-related internal state, with contact chemosensation of sex pheromones playing a key role in their activation.

This project will investigate the circuit mechanisms by which contact chemosensation induces the internal state change and promotes context-appropriate behaviours. Since male and female pheromones produce opposing behaviours (aggression vs courtship), they provide a tractable entrance to study internal states and their influence on produced behaviour.

I will use connectomics, behavioural assays, and functional imaging to explore how circuit elements induce the internal state and how different motifs facilitate appropriate behaviours. By integrating these results I will reveal how sensory inputs are transformed into adaptive responses depending on the social cues. This work bridges sensory perception and behavioural output, offering insights into the neural principles of social behaviour.

Introduction

During social behaviour organisms must continuously adapt their behaviour, carefully tailoring their actions based on cues such as the sex, location, age, or even species of their interaction partner. These social signals are integrated into internal states, which influence the likelihood of responding to a stimulus with a specific behaviour (Anderson, 2016; Berridge, 2004). This is a key feature of behaviour in many animals. Consider a human analogy, a knight navigating enemy territory. The knight's behaviour will be heavily shaped by internal states such as caution and alertness—motivated by a sense of danger. If they encounter another knight wearing a different surcoat, a visual cue associated with an unfamiliar allegiance, the knight might avoid any engagement to minimize risk. On the other hand, in his home castle, the same knight might readily approach someone wearing a surcoat of his own colors, motivated by familiarity and a sense of camaraderie. In both cases the knight's social behaviour is modified by an internal state. These internal states, which include phenomena like motivation, arousal, and emotion, are characterized by their persistence, scalability and their ability to bias perception toward context relevant stimuli (Anderson, 2016; Hindmarsh Sten et al., 2021; Schretter et al., 2024). Despite their importance, the precise neural mechanisms underlying the induction of such states remain poorly understood.

Courtship and mating behaviours offer an excellent framework for studying internal states in animal models, as they involve innate and robust behaviours with clearly defined targets (Anderson & Adolphs, 2014; LeDoux, 2012). For instance, male Drosophila exhibit a highly stereotyped courtship sequence when encountering a suitable female (Fig. 1a). This sequence includes, orienting towards the female, tapping of the female's abdomen with forelegs, vocalizations directed at the female, licking and finally attempted copulation (Yamamoto et al., 2014).

Drosophila melanogaster stands out as one of the most well-suited model organisms for studying the neural basis of behaviour. This is due to its extensively characterized genetics (Kohler, 1994; Lawrence, 1992; Rubin & Lewis, 2000), the availability of powerful genetic tools for neuroanatomical dissections (Brand & Dormand, 1995; Meinertzhagen et al., 2009; Pfeiffer et al., 2008), and the recent development of synaptically resolved whole-brain connectomes (Berg et al., 2025; Scheffer et al., 2020; Schlegel et al., 2024). Combined with the highly stereotyped courtship behaviour exhibited by male *Drosophila*, these resources make it an ideal system for investigating how sensory inputs modulate internal states in the context of courtship.

A key neuronal population implicated in courtship behaviour is the P1 cluster, a group of

fru-expressing neurons that are sufficient to drive male *Drosophila* courtship (Kohatsu et al., 2011; von Philipsborn et al., 2011; Yamamoto & Koganezawa, 2013). Artificial activation of P1 neurons induces courtship associated behaviours like following, wing extension, and song production, highlighting their role as a hub for integrating sensory cues contributing to the internal state of sexual arousal (Fig. 1b).

Interestingly, the internal state that regulates courtship when a male detects a female has close connections to an internal state regulating male-male aggression when the focal male detects another male. P1 neurons have been implicated in both behaviours, suggesting that a shared internal state facilitates behaviours of opposite valence (Anderson, 2016; Asahina et al., 2014; Hoopfer et al., 2015). This raises the question of how the selection between these behaviours is regulated. Assuming P1 neurons represent the internal state, they may disinhibit premotor neurons associated with courtship or aggression, allowing sensory sex-differentiating circuits to determine the behavioural outcome (Fig. 2).

My PhD thesis lab, in collaboration with others, is currently finalizing the male CNS connectome (Berg et al., 2025). This dataset provides a complete electron microscopy volume of the adult male *Drosophila* brain and nerve cord, allowing for a connectomic investigation of circuits connecting nerve cord and the central brain (Stürner et al., 2024) as well as the identification of sex differences by comparison with a new female brain connectome (Dorkenwald et al., 2024; Schlegel et al., 2024).

One such ascending circuit from the nerve cord to the brain is the contact chemosensory pathway, which transmits pheromone information detected during the male *Drosophila*'s tap behaviour. Two independent studies showed that the neuron types PPN1 (Kallman et al., 2015) and vAB3 (Clowney et al., 2015) relay contact chemosensory pheromone signals to the central brain, where they activate P1 neurons (Fig. 1b, f). Both studies propose a balanced excitation-inhibition circuit model in which excitatory inputs from PPN1 or vAB3 are counteracted by downstream inhibitory mAL neurons, ensuring precise regulation of P1 neuron activation (Fig. 2a).

The same studies (Clowney et al., 2015; Kallman et al., 2015) also identified F-cells as the sensory input neurons for the contact chemosensory pathway, providing upstream activation to vAB3 and PPN1. F-cells are pheromone-sensing neurons that selectively detect female pheromones and are defined by the coexpression of three DEG/ENaC subunits: ppk23, ppk25, and ppk29 (Pikielny, 2012). In contrast, male pheromone-sensing cells are characterized by the absence of ppk25 expression. Notably, M-cells have not been identified as part of the contact chemosensory processing pathway leading to P1 activation. If a balanced

excitation-inhibition mechanism regulates P1 activity, one would expect a connection of the M-cells to the inhibitory mAL neurons, yet such a link remains unconfirmed (Fig. 2b-d). Furthermore, Kohatsu et al. (2011) observed moderate activity in the lateral protocerebrum, the location of the P1 cluster, following male-to-male tapping (Fig. 1b). This suggests that PPN1 and vAB3 may serve different functional roles than previously assumed. Alternatively, it could point to the presence of additional circuit elements involved in male pheromone processing that have yet to be identified (Fig. 3d).

Recent work by my PhD thesis lab has identified candidates for F and M cells in the male CNS connectome (Berg et al., 2025). My preliminary analysis shows that the sensory inputs

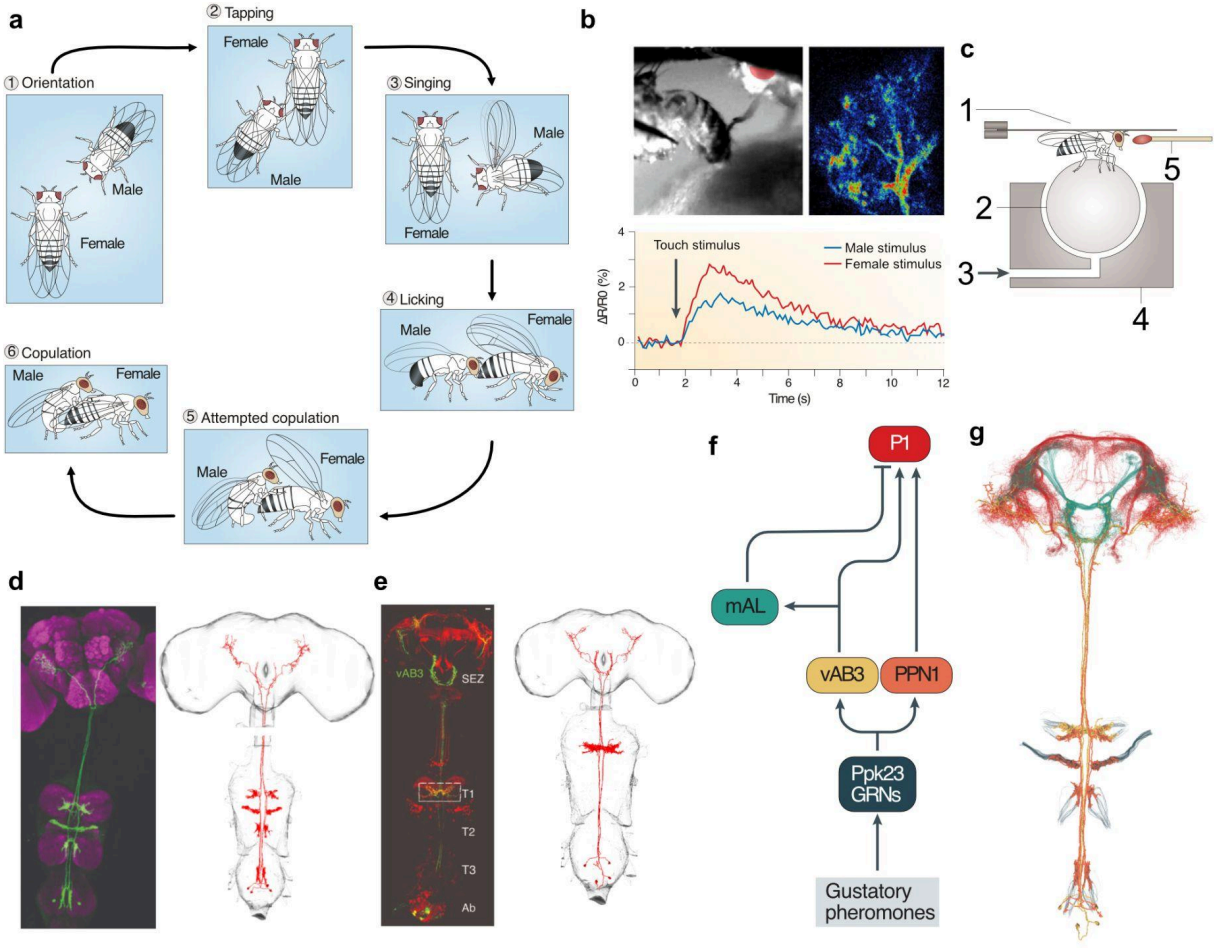

Figure 1: Contact chemosensation mediated signals induce an internal arousal state associated with courtship via the ascending neurons PPN1 and vAB3.

(a) Sequence of the stereotyped male courtship behaviour, adapted from Yamamoto & Koganezawa, 2013 **(b)** Stimulation by contact to male and female conspecific abdomens induces GCaMP activity in the the males protocerebrum, modified from Yamamoto & Koganezawa, 2013 and Clowney et al., 2015 **(c)** Schematic of spherical treadmill 1. Fly tether, 2. Styrofoam ball, 3. Air, 4. Ball holder, 5. Stimulus, adapted from Yamamoto & Koganezawa, 2013 **(d)** Comparison of light level PPN1 morphology to connectome morphology, light level image adapted from Kallman et al., 2015 (e) Comparison of light level PPN1 morphology to connectome morphology, light level image adapted from Clowney et al., 2015 **(f)** Schematic illustrating input from contact chemosensory pathway on P1, nodes are color coded to match cell type identity in (f), modified from Anderson, 2016 **(g)** Em morphologies of contact chemosensory pathway terminating at P1.

to vAB3 and PPN1 neurons do not overlap in cell type. This could be due to F cells inputting to PPN1/vAB3 having different morphology and connections, or it may suggest that PPN1 and vAB3 receive distinct sensory inputs and thus process different sensory signals.

Further work with colleagues in my PhD thesis lab has identified PPN1 and vAB3 neurons, as well as other neurons with PPN1/vAB3-like neuroanatomy (Fig. 1d, e, Fig. 3d), in the male CNS connectome (Berg et al., 2025). My preliminary analysis of how vAB3 and mAL neurons jointly connect to P1 has revealed a disparity in the vAB3/mAL input ratio across different P1 subtypes, along with a general overabundance of mAL inputs (Fig. 3a, c). This may suggest that only specific P1 subtypes process contact chemosensation. Additionally, my connectome analysis shows that only 0.5% of PPN1 output synapses directly target P1. Instead, the strongest postsynaptic partners are actually neurons intrinsic to the nerve cord, other ascending neurons similar to PPN1, and certain mAL subtypes, which also receive major input from vAB3.

This connectomic analysis of the contact chemosensory circuit raises several key unanswered questions. Do morphologically distinct neurons, which supposedly receive the same inputs, serve differential functions? Why do contact chemosensory neurons have such a vast diversity of overlapping and non-overlapping targets?

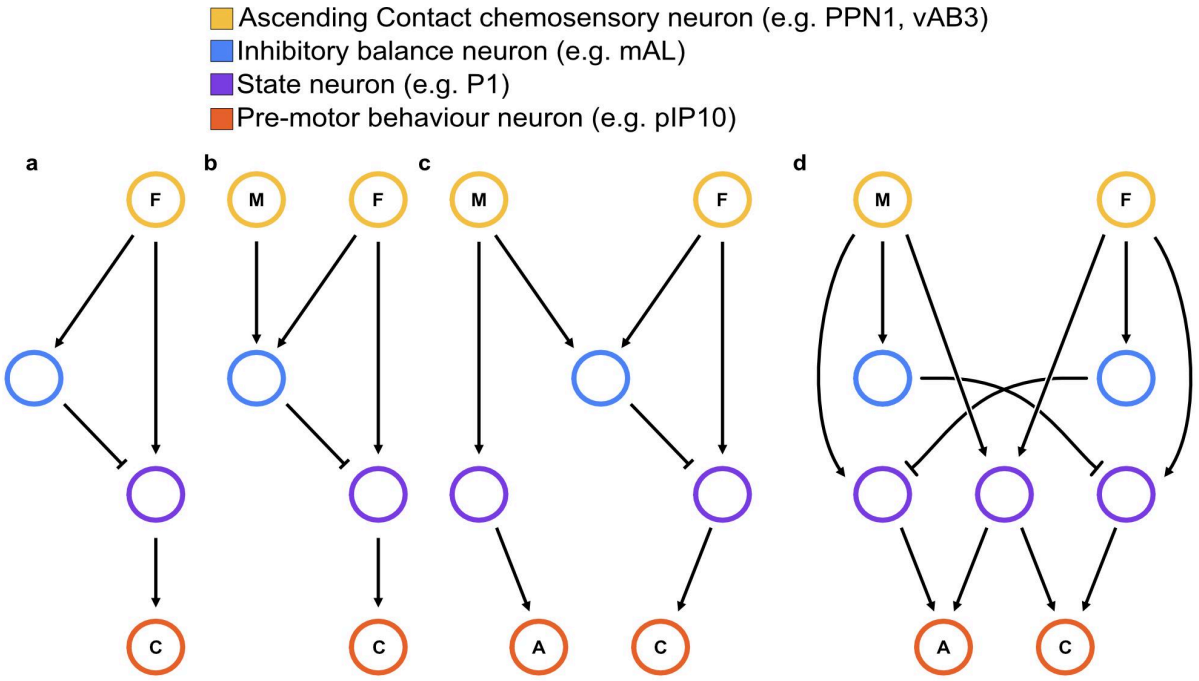

Figure 2: Hypothetical circuits illustrating how contact chemosensation may shape internal states to drive context-dependent behavior.

F = Female pheromone sensitive, M = Male pheromone sensitive, C = Upstream of courtship producing circuit, A = Upstream of aggression producing circuit **(a)** Balanced excitation-inhibition motif driving courtship as reported in Kallman et al., 2015 & Clowney et al., 2015 **(b)** Enhanced balanced excitation-inhibition motif driving courtship **(c)** Unilateral enhanced balanced excitation-inhibition motif driving courtship and aggression **(d)** Shared state motif driving courtship and aggression.

Assuming PPN1 receives input from male-pheromone-sensing M-cells, it could influence the excitation-inhibition balance regulating P1 (Fig. 2b). As mentioned earlier, I have found that PPN1 shows strong connections to the same mAL subclasses innervated by vAB3. This mechanism may contribute to the inhibition of courtship behaviour when male flies touch other males (Fig. 2b).

Male-male aggression is also mediated by P1 neurons (Anderson, 2016; Asahina et al., 2014; Hoopfer et al., 2015). Extending this balanced excitation-inhibition motif, it is possible that different P1 populations may integrate separate arousal states for courtship and aggression (Fig. 2c).

Such an arrangement would enable a male, upon tapping a female, to enhance his sexual arousal via courtship-related state neurons, while touching another male would inhibit these neurons and activate aggression-related state neurons. This configuration would support both an internal aggression state and an internal courtship state, but would also require a male-pheromone-sensitive contact chemosensation pathway and the existence of two distinct state neuron types (Fig. 2c).

The presence of females has been shown to increase the tendency of male *Drosophila* to exhibit aggressive behaviour (Anderson, 2016; Jung et al., 2020; Yuan et al., 2014), suggesting that aggression and courtship could share an internal arousal circuit facilitating both behaviours (Fig. 2d). In one hypothesis this circuit would comprise two context-specific state neurons, along with a shared state neuron (Fig. 2d). Cross-context inhibition—potentially mediated by mAL neurons—would allow female cues to modulate the shared internal state and thus promote the display of aggressive behaviours in response to male cues. This circuit would feature a combination of shared and distinct downstream targets of the ascending contact chemosensory neurons, a pattern observed in my preliminary connectomic analysis (Fig. 2a).

These specific circuit hypotheses propose various quite general mechanisms and motifs by which internal states may be regulated to enable context-appropriate behavioural output.

Objectives and Scope

In this project, I aim to investigate the pheromone detecting contact chemosensation circuit in the male Drosophila and how it produces the internal state change frequently associated with courtship of female conspecifics by males. Furthermore I want to examine how contact chemosensation and its associated neural circuitry distinguish the sex of a conspecific and how this subsequently influences the display of behaviours such as the courtship song or

aggression. This line of inquiry will provide insights into how sensory processing circuits produce internal states and experimentally demonstrate how these internal states drive appropriate behaviour.

Approach and Methodology

I aim to investigate the influence of contact chemosensation of internal state changes in male Drosophila across three levels, employing distinct techniques.

1. Connectome analysis

   Using the male CNS connectome (Berg et al., 2025), I will identify downstream and upstream target neurons of vAB3, PPN1, and mAL. This analysis will also explore potential subtypes within these cell classes that may differ in function or valence. Additionally this analysis will inform Objectives 2 and 3 by highlighting neurons to investigate in behavioural and imaging experiments.

2. Behavioural experiments

   I will combine behavioural assays with optogenetics in both unrestrained (freely walking) and restrained setups (spherical treadmill, Fig. 1c) to establish the behavioural significance of the identified target neurons in mediating courtship state changes.

3. Functional imaging

   I will use two-photon calcium imaging and genetically encoded calcium indicators in combination with a virtual reality setup (Fig. 1c) to study the functional connectivity and response dynamics of target cells to contact chemosensory stimuli.

Impact and Significance

This research will further our understanding of dynamic internal states that drive evolutionarily conserved behaviours. By investigating the initiation of the arousal state associated with courtship, triggered by either female or male pheromones, I aim to expand the current understanding of the circuit motifs driving internal state change. In doing so, my project will address a key question in neuroscience: how internal states are initiated and subsequently produce context relevant behaviour.

Specifically, my project will demonstrate how contact chemosensation facilitates courtship arousal in male *Drosophila* when they touch the abdomens of female conspecifics, while suppressing courtship when they touch other males. My preliminary analyses of the male CNS connectome (Berg et al., 2025) reveal a divergence from previously established models (Clowney et al., 2015; Kallman et al., 2015) suggesting a more nuanced and complex circuit

than previously understood. Leveraging *Drosophila* as a model organism, along with comprehensive existing connectomes, allows for the dissection and characterization of the entire circuit from sensory input to motor output.

Broader Implications

Ultimately, understanding the synaptically resolved neuronal architecture of this circuit and the underlying circuit motifs in *Drosophila* will provide a valuable framework for comparative connectomics. Contact chemosensation plays a crucial role in the courtship behaviour of all Drosophilids, with hydrocarbons differing between species and likely acting as a key driver of speciation (Khallaf et al., 2021; Seeholzer et al., 2018). This makes it an ideal model for studying how neural circuits and wiring patterns may evolve across time and species. By gaining a deeper understanding of how internal states are initiated and regulated, this research could also inform medical studies of more complex internal processes, such as attention and emotions, which are implicated in neurodevelopmental disorders like Autism Spectrum Disorder (Wiersema & Godefroid, 2018) and Attention Deficit Hyperactivity Disorder (Martino & Magioncalda, 2022).

Experimental strategy

1 Connectomic Analysis of the Contact Chemosensation Circuit and Its Impact on the Internal Arousal State of Male *Drosophila*

Understanding how contact chemosensation influences internal states requires a detailed understanding of the exact neural circuitry that transforms signals into state changes. Until now, there has been no resource to investigate these connections at a synaptic level. The complete electron microscopic volume of the adult male *Drosophila* brain and nerve cord provides a unique dataset that enables such investigations (Berg et al., 2025).

Previous studies have identified the contact chemosensory-sensitive neurons PPN1, vAB3, and their downstream partner mAL as key players in activating the courtship neurons P1 (Clowney et al., 2015; Kallman et al., 2015). My preliminary connectomic analysis has shown that the overlap in targets between mAL, PPN1, and vAB3 neurons is limited (Fig. 3a), suggesting that an excitation-inhibition balance alone (Fig. 2a) is unlikely to be the primary mechanism driving state changes (Clowney et al., 2015; Kallman et al., 2015).

The male CNS connectome (Berg et al., 2025) will allow me to further explore the organization of this circuit and allow me to identify critical neurons that induce the arousal state change in male *Drosophila*. Through this connectomic analysis I plan to to inform aim 2 and 3 by generating circuit hypotheses (Fig. 2) to select existing and generate new sparse driver lines using the Gal4-UAS and LexA-LexAop dual expression systems to conduct functional interrogation (Brand & Perrimon, 1993; Lai & Lee, 2006; Meissner et al., 2025).

1.1 Subtype-Specific Roles in the vAB3-mAL-P1 Circuit During Contact Chemosensation

Previous studies have suggested that the sexually dimorphic circuits of the ascending vAB3 neurons and the brain-internal mAL neurons form a parallel convergent circuit onto P1 courtship neurons (Fig. 2a) (Clowney et al., 2015). It is proposed that the excitatory input from vAB3 and the inhibitory input from mAL together precisely regulate the internal courtship state.

My PhD thesis lab has identified vAB3 and mAL neurons in the male CNS connectome (Fig 2d, e) (Berg et al., 2025). The morphologies and connections of these neurons indicate the existence of several subtypes targeting different downstream P1 neurons (Fig. 3a). My preliminary analysis shows that while vAB3 and mAL partly co-innervate specific subtypes of P1 neurons, other mAL neurons downstream of vAB3 target additional P1 subtypes (Fig. 3a). As a result, different P1 subtypes display type-specific ratios of mAL and vAB3 input (Fig. 3c), suggesting that these neurons play distinct roles in modulating the internal state.

In my future connectome analysis, I plan to further investigate the differences between the vAB3 and mAL input pairs and identify which pairs are essential for changes in the internal state. My PhD thesis lab and collaborators have already generated various Gal4-UAS lines that target specific subsets of mAL, enabling me to functionally interrogate the role of mAL in regulating the internal arousal state of male *Drosophila*.

1.2 Mapping the PPN1 Contact Chemosensation Circuit and Its Role in Internal State Change

Similar to vAB3 and mAL, the ascending neuron type PPN1 has been implicated in a contact chemosensation circuit that operates on an excitation-inhibition balance motif (Kallman et al., 2015). This circuit ultimately activates the P1 courtship neurons.

PPN1 is a fruitless-negative neuron, a marker commonly linked to sexually dimorphic neurons (Demir & Dickson, 2005). This indicates that it may be a contact chemosensation neuron detecting conspecific sex which is present in male and female flies (Cachero et al., 2010). To validate this hypothesis, I plan to compare the morphology and connectivity of male and female PPN1s leveraging the available female connectomes (Dorkenwald et al., 2024; Schlegel et al., 2024), alongside the male CNS connectome (Berg et al., 2025). This analysis will assess whether PPN1s function is related to courtship behaviour or if it works as a more general sex detecting cell.

Interestingly, my preliminary connectome analysis suggests that, although PPN1 activation leads to P1 activation (Kallman et al., 2015), PPN1 only sparsely forms direct connection with P1. Instead, PPN1 exhibits strong connections to mAL and to intrinsic nerve cord neurons. This indicates a circuit motif distinct from parallel processing (Fig. 2b, c).

Moving forward, I will identify PPN1's downstream targets to determine how its activation ultimately leads to P1 activation. Additionally, I aim to uncover the role of nerve cord intrinsic neurons in processing contact chemosensory information from PPN1.

1.3 PPN1 and vAB3-Like Neurons and Their Connectivity in the Contact Chemosensation Circuit

While my PhD thesis lab has identified PPN1 and vAB3 neurons in the male CNS connectome (Fig. 1d, e) (Berg et al., 2025), my preliminary analysis has uncovered additional neuron types with similar projection patterns and synaptic partners (Fig. 3d). These neurons often differ from the identified cells only in minor aspects, such as the absence of single branches or variations in soma location.

Analysis of these candidate neurons reveals interconnectivity with the previously identified PPN1 and vAB3 neurons, as well as their downstream targets, suggesting a potential role in

contact chemosensation. For example, the neuron type AN05B035 (Fig. 3d) exhibits strong reciprocal connectivity with vAB3 neurons. Moreover, some other candidate neurons establish indirect connections between PPN1 and vAB3 neurons. For instance, the PPN1-like AN05B023 neuron (Fig. 3d) serves as an upstream partner to both PPN1 and vAB3.

In my ongoing connectomic analysis, I aim to investigate whether these neurons represent subtypes of the already identified types or constitute functionally distinct types by investigating their other upstream and downstream partners.

1.4 Mapping of Sensory Input Neurons to PPN1 and vAB3

Previous studies have identified M-cells, defined by expression of the ENaC channel ppk23, and F-cells, defined by their co-expression of ppk23 and ppk25, as the sensory leg bristle neurons involved in pheromone detection of the contact chemosensation circuit (Clowney et al., 2015; Kallman et al., 2015; Thistle et al., 2012; Vijayan et al., 2014).

My PhD thesis lab has identified candidate neurons for these M- and F-cells within the male CNS connectome (Berg et al., 2025). My preliminary connectomic analysis revealed low connectivity between these candidates and PPN1 and vAB3 neurons. Additionally, my analysis indicates that PPN1 and vAB3 receive minimal input from shared chemosensory cells (Fig. 3b), suggesting that these neurons may process separate sensory signals, potentially related to male or female pheromones.

To identify the sensory neurons associated with PPN1 and vAB3, I plan to analyze light-level

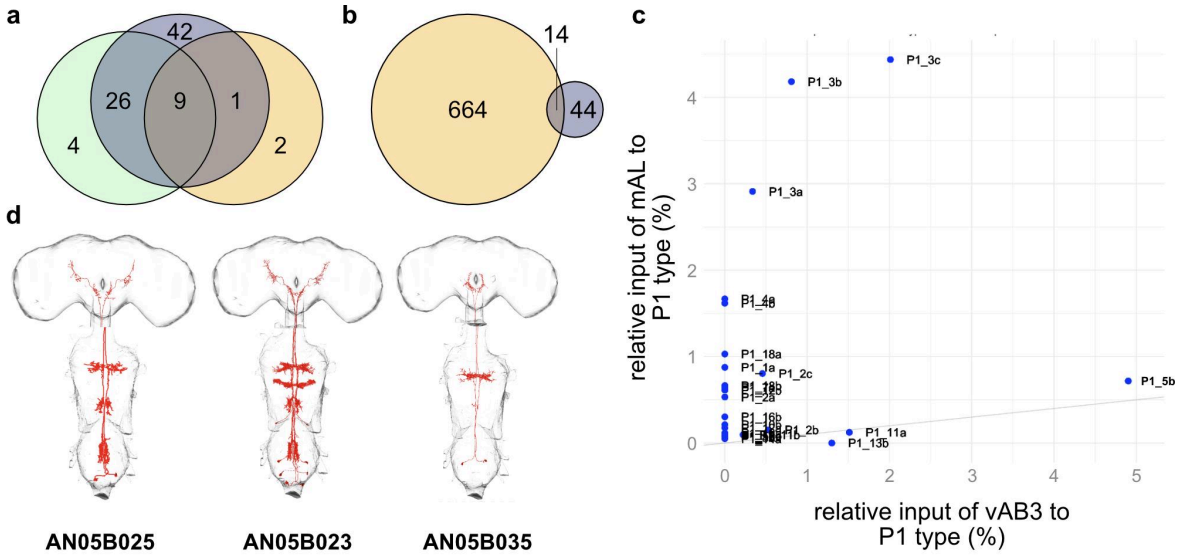

Figure 3:Morphologically and connectively distinct P1 receive distinct contact chemosensation input.
(a) Venn diagram showing the overlap of unique P1 targets of vAB3 (green), PPN1 (orange), and mAL (blue) **(b)** Venn diagram showing the overlap of unique inputs of PPN1 (orange) and vAB3 (blue) **(c)** Scatter plot showing the relative contributions of vAB3 target neurons and their downstream mAL neurons to P1 input across different P1 subtypes **(d)** EM morphologies of three ascending neurons with similar structure and connectivity to PPN1 and vAB3, suggesting their involvement in contact chemosensation.

images of specific driver lines marking cells expressing either ppk23 or both ppk23 and ppk25. These morphologies will be compared to those in the male CNS connectome.

If these driver lines lack sufficient resolution, I will employ transsynaptic labeling (Talay et al., 2017) in combination with sparse driver lines for PPN1 and vAB3 to identify their presynaptic sensory cells and match them to existing EM morphologies. To further characterize these presynaptic cells, I will investigate whether they express ppk23, ppk23/ppk25, or other pheromone receptors such as Gr32a or GR68a (Kohatsu et al., 2011; Shankar et al., 2015; Wang et al., 2011). For this, I will use RNA in situ hybridization to detect the expression of target pheromone receptors (Choi et al., 2018; Wang et al., 2011). By integrating connectomic analysis with molecular methods, I aim to identify the precise sensory inputs involved in contact chemosensation.

2 Behavioural Testing of Connectome-Based Circuit Hypotheses

In male *Drosophila*, contact between the sensory neuron-containing forelegs and the abdomen of a female conspecific induces an internal state change of arousal. This state is characterized by the male closely following the female, extending his wings, and producing courtship songs (Fig. 1a) (Kohatsu et al., 2011; Yamamoto et al., 2014).

My preliminary connectomic analysis has identified various subtypes among the PPN1, vAB3, and mAL neurons associated with this state change. To test the behavioural relevance of these neurons I will use behavioural assays which assess the males courtship behaviour towards a courtship target. The focal animals will be transgenically modified using the Gal4-UAS and LexA-LexAop dual expression systems to sparsely express optogenetic and thermogenetic tools to activate or silence specific neuron types (Abdelfattah et al., 2019; Boyden et al., 2005; Brand & Perrimon, 1993; Döring et al., 2002).

Through this approach I will be able to identify neuron types and populations crucial to the contact chemosensation mediated internal state change and the resulting behaviour.

2.1 Testing Connectome-Based Hypotheses Through Optogenetic and Thermogenetic Manipulations in Freely Walking Flies

In this part of the project, I will use specific driver lines to ectopically express optogenetic tools for artificial manipulation in the neuron types identified in aim 1 (Boyden et al., 2005; Döring et al., 2002; Hamada et al., 2008). These tools will allow for precise activation or inhibition of neuronal subpopulations, enabling me to test connectome-based hypotheses regarding their roles in behaviour. To assess the effects of these manipulations, I will employ computer vision-based pose estimation algorithms (Mathis et al., 2018) to analyze changes in

courtship behaviour, particularly the following behaviour associated with courtship (Fig 1a).

Experiments will be conducted in a mating assay where flies are free to move within an arena, pairing a genetically modified male fly with a wild-type female fly. By modifying the female's cuticular hydrocarbon profile and removing the pheromone producing oenocytes (Billeter et al., 2009), I will be able to manipulate the pheromone cocktail detected by the male's contact chemosensation circuit, enabling the identification of circuit elements associated with specific chemosensory cues.

The selection of neuronal subpopulations for these experiments will be guided by the results of my previous analysis. For instance, if the connectome suggests that distinct vAB3 subtypes play a critical role in the internal state change, I could express CsChrimson in the vAB3 to test whether optogenetic activation is sufficient to induce courtship behaviour. I will test both male and female conspecifics or a moving magnet, a target wild type flies usually don't court, but which external P1 activation can induce courtship toward (Seeholzer et al., 2018). Discovering such a subpopulation and the corresponding sparse driver line would break the underlying circuit open and would allow for easy manipulation of the flys arousal state.

2.2 Testing Connectome-Based Hypotheses Through Optogenetic and Thermogenetic Manipulations in Tethered Flies

In *Drosophila* contact chemosensation studies, the most compelling functional experiments have been performed using a spherical treadmill setup (Fig. 1c) (Clowney et al., 2015; Kohatsu et al., 2011). This setup involves tethering a fly with a wire and placing it on a styrofoam ball suspended on a constant airstream, effectively allowing the fly to "walk in place" (Loesche & Reiser, 2021; Seelig et al., 2010).

The ball's movements are tracked to provide precise measurements of the fly's walking direction thus providing a courting index of how well the focal fly is aligned with presented stimuli (Moore et al., 2014). This setup offers tight experimental control and allows me to perform the behavioural experiments previously described for neurons that show a functional impact on behaviour with higher fidelity. In addition this setup is compatible with functional two-photon imaging making it an excellent intermediate approach between the behavioural assays described in aim 2 and the functional imaging experiments planned for aim 3.

3 Revealing the Neuronal Activity Facilitating Contact Chemosensory-Mediated Internal State Change in Male *Drosophila*

At this stage of the project, the previous two aims will have identified the relevant circuit nodes and motifs that induce contact chemosensation mediated internal state changes and the

associated behavioural output. To investigate the mechanism of how these circuit elements produce the observed state changes at the single-cell level, I will perform functional imaging on transgenic flies expressing genetically encoded activity reporters and optogenetic neuromodulators. This will be conducted under a two-photon microscope paired with the spherical treadmill setup described in Aim 2.2 (Fig. 1c).

By combining targeted manipulation and simultaneous imaging of previously identified circuit elements responsible for the arousal change of male *Drosophila*, this approach will provide critical insights into how internal states are produced and how they influence the display of context relevant behaviour.

3.1 Functional Imaging of Sensory Neurons

While PPN1 and vAB3 are both key neurons involved in contact chemosensation, PPN1 innervates all three thoracic segments (Fig. 1d), suggesting that it receives input from contact chemosensory cells across all legs (Kallman et al., 2015). As outlined in Aim 1.4, I aim to identify the sensory inputs to the ascending neurons PPN1 and vAB3.

Complementary to the methods outlined in Aim 1.4, I will present natural male or female pheromone cocktails, as well as their isolated components, to individual legs while selectively expressing and imaging genetically encoded calcium indicators in ppk23+/ppk25- or ppk23+/ppk25+ sensory cells. This approach will identify which legs and sensory neurons are sensitive to the presented pheromones. I will then compare the responsive sensory cells to those annotated in the connectome, refining their identities within the male CNS connectome (Berg et al., 2025). In parallel, I will selectively express and image genetically encoded calcium indicators in PPN1 neurons while stimulating each leg individually. Intersecting the data from sensory neuron imaging and PPN1 neuron imaging, will allow me to determine which legs contribute to contact chemosensation and whether PPN1 exhibits a specialized role, such as male-specific pheromone detection. To generalize these findings, I will replicate this approach for vAB3 neurons and their subpopulations to uncover potential differences in pheromone mapping between these two morphologically distinct neuron types (Fig. 1e, d).

This systematic approach will allow me to determine if differing sensory input to distinct circuit elements enables sexual discrimination within the contact chemosensation circuit facilitating the internal state change of arousal in male *Drosophila*.

3.2 Functional Imaging of Second and Third Order Contact Chemosensory Neurons

The currently proposed mechanism of how contact chemosensation induces an internal state change that results in courtship behaviour involves a balance of inhibition and excitation,

facilitated by excitatory ascending neurons (PPN1 and vAB3) and inhibitory mAL neurons (Fig. 2a) (Clowney et al., 2015; Kallman et al., 2015). However, this hypothesis assumes that the 148 P1 neurons form a functionally unified type.

My preliminary analysis, which treats P1 neurons as a supertype comprising morphologically and connectively distinct subtypes, reveals that mAL, PPN1, and vAB3 neurons only partly converge on the same nodes (Fig 3a, c). In this part of my project, I aim to leverage optogenetic and thermogenetic activation and inhibition, alongside genetically encoded activity indicators, expressed in sparse neuronal subpopulations, to identify the circuit logic underlying the internal state change in male *Drosophila* (Fig. 2).

By this stage, I will have identified a genetic driver line that recapitulates, when optogenetically stimulated, the internal state change associated with the male fly's tap behaviour towards a virgin female fly. Combining this activation with the expression of genetically encoded activity indicators in downstream neurons, identified in the previous aims, will allow me to observe the distributed activity throughout the circuit, which ultimately leads to the internal state change and the display of courtship behaviours.

To determine whether specific connections between downstream nodes are critical to production of an internal state change, I will express inhibitory neuromodulators such as Kir2.1 (Döring et al., 2002), or GtACR (Mohammad et al., 2017), in order to establish a minimal circuit model for arousal. As a readout, I will either use a neuron from the P1 population, the song-inducing pIP10 neuron (von Philipsborn et al., 2011), or ideally the output of natural courtship behaviour.

These functional interrogations will be conducted using a two-photon microscope to image neural activity paired with a spherical treadmill to record behavioural responses (Fig. 1c). This setup will allow me to detect stereotypical courtship behaviours in response to artificial activation/inhibition. Additionally, to complement artificial activation with natural stimuli, I will present thoraxes of flies with various natural pheromone cocktails.

By integrating these results with my connectomic and behavioural analyses, I aim to clarify the mechanisms of how contact chemosensory cues modulate an internal arousal state in male *Drosophila*. This will reveal how sensory inputs are transformed into context appropriate behavioural responses, such as courtship or aggression. Just as a knight adapts to an approaching figure's surcoat, male *Drosophila* interpret pheromonal cues to navigate social interactions. This work bridges sensory perception and behavioural output, offering insights into the neural principles governing social behaviour.

Work schedule

I have already begun the connectomic analysis of how the function of PPN1, vAB3, and the downstream mAL may differ using the male CNS connectome (Berg et al., 2025). Furthermore, I have started identifying additional ascending and nerve cord internal neurons that, based on connectivity, are involved in the contact chemosensation circuit. My planned timeline for future experiments is outlined below (Fig. 4). My previous training in neuroscience techniques includes computational analysis of cell activity data, as well as two-photon imaging and the modification of such systems to enable the simultaneous monitoring of behavioural outputs. I also have experience with RNA in situ hybridization. Since I worked with zebrafish during my master's, I have been adapting to Drosophila as my model system and have already been introduced to basic tasks such as fly handling and brain dissections. I plan to apply to the Cold Spring Harbor Laboratory course "Drosophila Neurobiology: Genes, Circuits & behaviour" which would train me in the techniques commonly used in Drosophila neuroscience.

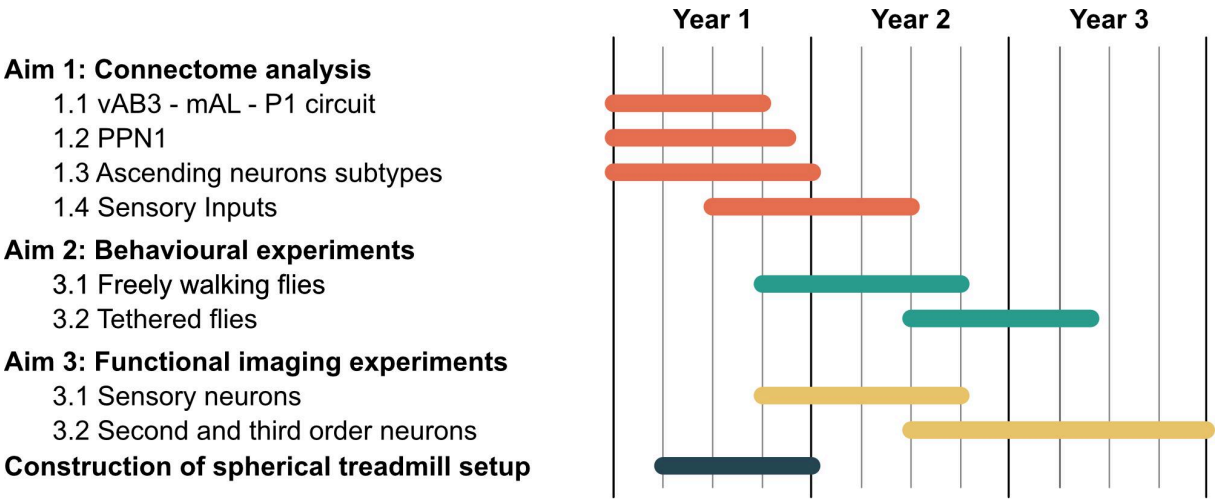

Figure 4: Gantt chart representing planned experiment and work schedule.

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Summary of MSc thesis

Mapping functional dynamics and neuronal structure in a hindbrain neural integrator circuit
Supervisor: Prof. Dr. Armin Bahl

Animals constantly need to process noisy information gathered from their natural environment. To extract relevant information and perform appropriate behavioural output in response to these noisy inputs, neurons are organized into neural circuits which compute responses to the presented information. To understand the sensorimotor transformations performed in such biological neural networks, it is necessary to understand their components and how they subsequently interact. Previous work by Prof. Dr. Bahl established a biophysically plausible model of such a circuit processing noisy motion evidence in the anterior hindbrain of the larval zebrafish (Bahl & Engert, 2020).

During my master's thesis I further investigated the functional dynamics and localization of the neuronal clusters constituting this motion evidence integrator. Furthermore, I combined functional investigations with the morphological mapping of the circuit establishing the projection patterns as well as the neurotransmitter identities of the different neuron classes involved to investigate the architecture of the larval zebrafish motion integrator.

Using functional two-photon calcium imaging (Fig. 1a) combined with targeted single cell photoactivation and *in situ* fluorescent rna hybridization (Fig. 1b) I was able to determine the activity, morphology and neurotransmitter identity of the different cell classes constituting the circuit (Fig. 1c-d). Based on the morphologies of the cell types I was able to split the integrator type into two subclasses either projecting contralateral or ipsilateral (Fig. 1c,d). This established a close structure to function relationship with each cell type (ipsilateral integrator, contralateral integrator, dynamic threshold, and motor command) exhibiting characteristic morphological features.

To further validate this neuron stereotypy I co registered my cells to a zebrafish reference brain (Randlett et al., 2015) to compare my photoactivated morphologies to the electronimcroscopically reconstructed neurons in an existing correlated light and electron microscopy volume (CLEM) of the same circuit. Through this I was able to extract and identify 7 characteristic morphometrics (Fig. 1g) which suffice to reliably predict (F1-score = 0.794) the functional class of CLEM-neurons using a linear discriminant analysis classifier (LDA) trained only with the photoactivated cell morphologies (Fig. 1h).

Together these results identify the architecture of the larval zebrafish evidence accumulator and establish a function to structure stereotypy in a vertebrate brain.

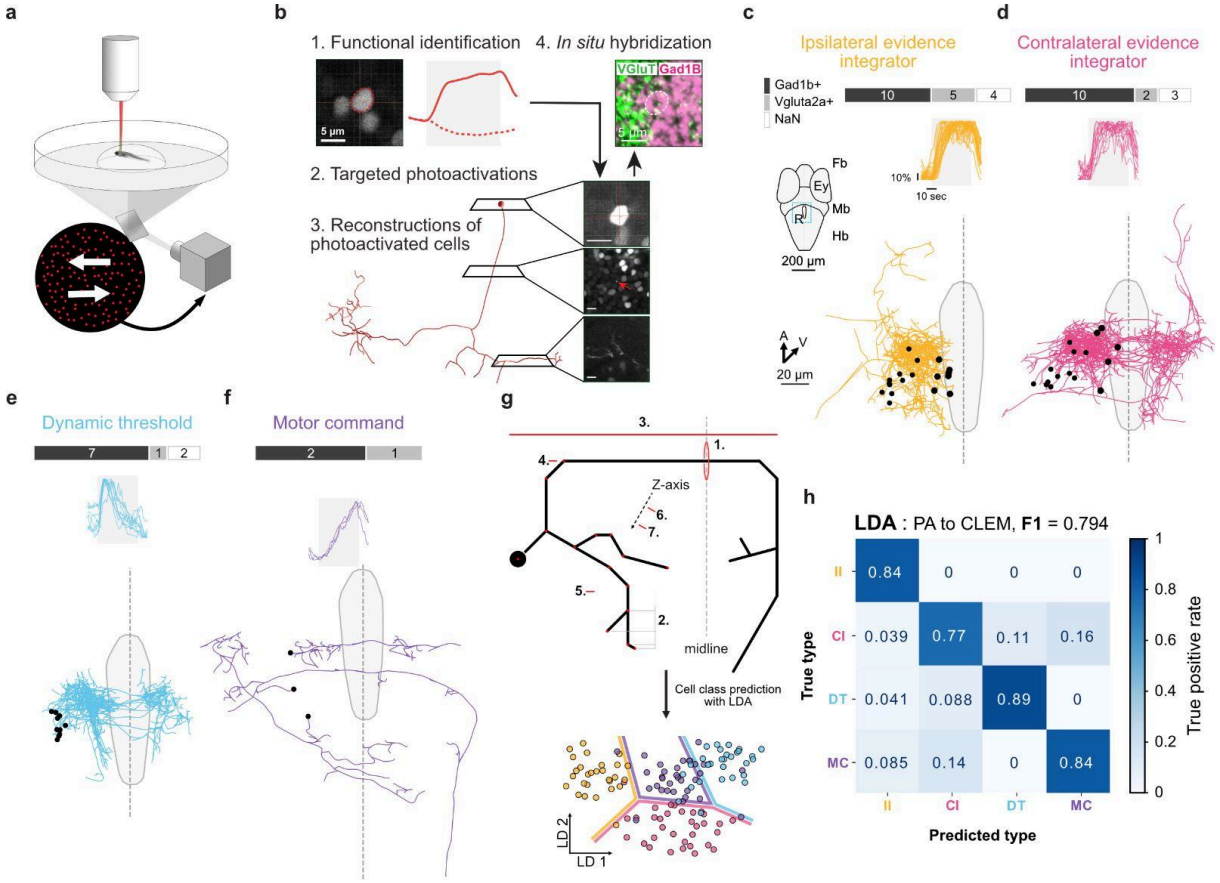

Figure 1: Functionally guided single cell photoactivation establishes a structure to function stereotypy within the larval zebrafish motion integrator.

(a) Schematic of experimental setup under two-photon microscope. Fully agarose-embedded 5 dpf transgenic zebrafish larvae are presented with random-dot-motion showing motion to the left or the right. **(b)** Schematic of the functionally guided photoactivation process. (1) Functional imaging to identify cells with responses in line with (Bahl & Engert, 2020). (2) Photoactivation of identified cells at 760 nm. (3) Volumetric scan of the photoactivated morphology and subsequent reconstruction. (4) HCR RNA-FISH stain for gad1b-RNA (associated with GABA synthesis) and vglut2a-RNA (associated with glutamate trafficking). Staining imaged in the same microscope and target cells cross identified. **(c)** Structure-function relationship of functionally imaged and ipsilateral integrator. Top: distribution of neurotransmitter identity shown as Gad1b-positive (black), Vglut-positive (gray), and not identifiable (white). Left: schematic showing coarse brain organization (dorsal view) and location of the reconstructed neurons below (green dashed square) surrounding outlines of the raphe (also shown below). Right: traces representing the normalized neuronal activity over time. Bottom: reconstructed ipsilateral evidence integrators with somas in black and neurites in orange. Dashed line represents the midline. **(d-f)** shows identical representation for other neuron types. All neurons are registered to a reference brain (Randlett et al., 2015). Abbreviations: Mb, midbrain; Hb, Hb; R, raphe; Ey, eye; A, anterior; V, ventral. **(g)** Schematic of the features selected to predict functional types and subsequent prediction in LDA space. 1. Bilaterality, 2. Number of ipsilateral nodes, 3. Width, 4. Minimum ipsilateral Y coordinate, 5. Average Y coordinate, 6. Average Z coordinate, 7. Minimum ipsilateral Z coordinate **(h)** Confusion matrix of functional type prediction of CLEM neurons with an LDA classifier trained on photoactivated neuron morphologies. Matrix shows a true positive rate. F1-score = 0.794.

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Reasons to work with the group

The Jefferis group aims to decode how neural circuits process chemosensory information and transform it into behavior. My project focuses on dissecting how contact chemosensation contributes to the internal arousal state present in male Drosophila and how this internal state produces context-relevant behaviour. This aligns closely with the group's expertise in sensory processing, circuit mapping, and the transformation of olfactory and pheromonal cues into behaviorally relevant representations.

Additionally, the Jefferis group collaborates closely with the Drosophila Connectomics Group at the University of Cambridge. Together, they have proofread and annotated several connectomes and developed computational methods to identify functionally relevant circuits and circuit nodes. Since my project relies heavily on the male Drosophila CNS connectome, the group's experience in leveraging connectomics for circuit dissection will be invaluable in refining my analyses and integrating them with my functional experiments.

The Jefferis group is located at the MRC Laboratory of Molecular Biology, which offers an exceptional environment for scientific growth. The collaborative atmosphere encourages the exchange of ideas, allowing me to engage with researchers from closely related fields as well as those working on entirely different topics. This multidisciplinary setting will provide fresh perspectives and insights, further benefiting my PhD project.

Beyond connectomics, the Jefferis group is an excellent lab for experimental investigations of neuronal circuits. Their expertise in molecular techniques, including histological staining and genetic manipulation of the Drosophila genome, as well as their proficiency in brain dissection—from central brain regions to the entire nervous system—will allow me to quickly adopt and apply the rich neuroanatomical toolkit available in Drosophila research.

For behavioral and imaging experiments, the Jefferis group employs a range of setups, from behavioral arenas compatible with optogenetics to two-photon microscopy for functional imaging. This combination of connectomic, behavioural, molecular, and imaging approaches provides an ideal environment for me to succeed in my PhD and investigate the neural mechanisms underlying contact chemosensation and internal states in Drosophila.